Accepted Manuscript

Molecular characterization of Sarcocystis spp. in intestine mucosal scrapings and fecal samples of Pampas fox (Lycalopex gymnocercus)

Nathalia Paula Scioscia, María Laura Gos, Guillermo María Denegri, Gastón Moré

PII: S1383-5769(17)30145-9
DOI: doi: 10.1016/j.parint.2017.06.004
Reference: PARINT 1688

To appear in: *Parasitology International*

Received date: 21 March 2017
Revised date: 8 May 2017
Accepted date: 11 June 2017

Please cite this article as: Nathalia Paula Scioscia, María Laura Gos, Guillermo María Denegri, Gastón Moré , Molecular characterization of Sarcocystis spp. in intestine mucosal scrapings and fecal samples of Pampas fox (Lycalopex gymnocercus). The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Parint(2017), doi: 10.1016/j.parint.2017.06.004

Molecular characterization of *Sarcocystis* spp. in intestine mucosal scrapings and fecal samples of Pampas fox (*Lycalopex gymnocercus*)

Nathalia Paula Scioscia[1,2]; María Laura Gos[2,3]; Guillermo María Denegri[1,2]; Gastón Moré[2,3]

1-Instituto de Investigaciones en Producción, Sanidad y Ambiente (IIPROSAM), Facultad de Ciencias Exactas y Naturales, UNMdP. Funes 3350, Mar del Plata, Argentina.

2-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

3-Laboratorio de Inmunoparasitología, Facultad de Ciencias Veterinarias, UNLP. Calle 60 y 118, La Plata, Argentina.

Abstract

Sarcocystis spp. are obligatory intracellular protozoan parasites which can infect humans and animals. Most of *Sarcocystis* species were identified based on the detection of muscle cysts in different intermediate hosts (IH). Regarding to natural infection in definitive host, there are few reports which have reached to determining species of *Sarcocystis*. The present work was aimed to studying the occurrence of *Sarcocystis* spp. (oocysts and sporocysts) in mucosal scrapings of small intestine and fecal samples of one the most abundant wild canids from South America, *Lycalopex gymnocercus* (Pampas fox), and to identify the *Sarcocystis* spp. using molecular tools. A total of 131 free-living *L. gymnocercus* were sampled in rural areas located in several departments from Buenos Aires province, Argentina. Fecal samples from all the animals and 33 small intestines were analyzed. Fecal and mucosal scrapings samples were analyzed by sugar flotation method and once oocysts or sporocysts were detected, sedimentation was performed and DNA extracted with a commercial kit. A PCR was conducted using primers targeting a fragment of the 18S rRNA gene and the amplicons were purified and sequenced. Of the total Pampas foxes analyzed, 23 (17.6%) had *Sarcocystis* spp. oocysts/sporocysts in fecal and/or mucosal samples. *Sarcocystis* spp. sporocysts were detected in 13.0% (17/131) of fecal samples and in 39.4 % (13/33) of mucosal samples by the initial sugar flotation. Twenty one *L. gymnocercus* samples were processed by DNA extraction and PCR. Molecular identification of *Sarcocystis* spp. infection was successfully achieved in 14 foxes and was distributed as follows: 4.6% *S. cruzi* (6/131), 3.8% *Sarcocystis* spp. using birds as IH (*S. albifronsi* and *S. anasi* among others, 5/131), 0.8% *S. tenella* (1/131) and 1.5% (2/131) with low homology (97%) with *S. miescheriana*. In one fecal sample with spherical oocysts, the sequencing results showed a 100% sequence identity with *Hammondia heydorni*. The results show that the mucosal scrapings are the eligible sample to identify prevalence and to proceed with species identification. *Lycalopex gymnocercus* is suggested as definitive host for *S. cruzi*, *S. tenella* and probably various *Sarcocystis* spp. using birds as intermediate hosts as well as for *H. heydorni*.

Keywords: *Sarcocystis* spp.; wild canid; fecal samples; mucosal scraping; sequencing; definitive host.

1. Introduction

Protozoans of the genus *Sarcocystis* (phylum Apicomplexa) are one of the most frequent parasites of warm blooded (including man) and poikilothermic animals worldwide. Generally, these protozoans have an obligatory two-host, prey-predator life cycle. Usually, carnivorous or omnivorous are the definitive hosts (DH) and the sexual development occurs in the small intestine of the predator (both gametogony and sporogony stages). Sporulated oocyst or free sporocysts, released into the intestinal lumen, are passed in feces and are excreted over a period of several months. Intermediate hosts (IH), typically herbivores or omnivores, become infected by ingesting sporocysts via food or water. The asexual reproduction typically occurs in vascular endothelial cells (schizont stage) and striated muscle cells (sarcocyst stage) of the IH. The DH becomes infected by ingesting IH tissues with mature sarcocysts. The morphology of the oocyst and sporocysts from most of *Sarcocystis* spp. is similar; therefore microscopy has little or no taxonomic value in contrast to other coccidian species. Traditionally, experimental infection has been used to identify the role as *Sarcocystis* spp. DH of carnivores or omnivores [1]. On the other hand, molecular methods have been arising as important tools to minimize animal experiments. The variable regions of the 18S rRNA gene have been shown to be a good genetic marker to characterize *Sarcocystis* spp. [2, 3, 4]. There are several reports of wild canids with natural intestinal infections with *Sarcocystis* spp. but only a few studies came to the species determination [1, 3, 5].

As a part of the biocoenosis, wild canids participate in the spread of various parasites [6], and could be DH of various *Sarcocystis* spp. [1]. Although *Sarcocystis* spp. are rarely pathogenic to the DH, acute infection in the IH may cause extensive tissue damage and subsequent increased mortality and economical lost [1, 7].

The Pampas fox, *Lycalopex gymnocercus*, is the most abundant wild canid from South America and inhabits grasslands and open woodlands. It is a generalist and opportunistic mesopredator and the dietary items vary according to seasonal availability and geographic location [8, 9]. Parasitism is often heavily influenced by the ecology of the host, as the parasite will have adapted its life cycle according to host diet, behaviour and habitat [10]. Regarding to natural *Sarcocystis* spp. infection in canids as DH, there are few reports infection from South America, mainly in dogs [e. g. 11]; besides none studies have reached to determining species of *Sarcocystis* present. *Sarcocystis* spp. using Pampas fox as DH have been not identified; however since is a wide distributed carnivore in South America, *Sarcocystis* species producing cysts in the ingested prey species are highly probable [12].

The present work was aimed to studying the occurrence of *Sarcocystis* spp. (oocysts and sporocysts) in mucosal scrapings of small intestine and fecal samples of *L. gymnocercus*, and to identify the *Sarcocystis* spp. using molecular tools.

2. Material and methods

2.1 Samples

The study was conducted in rural areas located in seven departments (*Azul, Benito Juarez, Lobería, Necochea, Carmen de Patagones, Tandil and Villarino*) from Buenos Aires province, Argentina. This area is currently dominated by cattle farming and agricultural activities and it is home

to high densities of *L. gymnocercus*. Pampas foxes were collected as part of an eco-epidemiological study of endoparasites ofthe gastrointestinal tract in this fox species.

In this study a total of 131 free-living *L. gymnocercus* were analyzed (67 females and 64 males). Most of these animals were found dead along road ways (n=80) and the others (n=51) were recovered during the commercial hunting season authorized in Buenos Aires. Fecal samples (content of the rectum) from all the animals and 33 small intestines were analyzed. The samples collection and transport to the laboratory was allowed by the *Ministerio de Asuntos Agrarios and Dirección de Flora y Fauna of Buenos Aires*. All Pampas foxes were adults. The samples were kept at −20◦C for at least 1 month prior to processing.

2.2 Microscopic analysis

Fecal samples were analyzed for protozoan stages (oocysts/sporocysts) identification using a sugar flotation method (Sheather flotation, [13]) and subsequently examined microscopically (by duplicate).

Small intestines were thawed and mucosal scrapings from the posterior two thirds were obtained by using an individual glass slide for each sample. The collected material was transferred to a 50 ml tube and conserved at −20◦C until used. Initially, 1.5 g of each mucosal scraping was homogenized with 15 ml of sucrose solution and centrifuged at 500g during 10 min and processed as previously. Once oocysts/sporocysts were detected, the remaining material of the sample was concentrated by overnight water sedimentation, followed by sucrose flotation and concentration by water sedimentation and conserved for further studies as described previously [3].

2.3Molecular analysis

DNA was extracted from aliquots (around 300 µl) of the concentrated material using a commercial kit (ZR fecal DNA Zymo research), according to manufacturer's instructions. A fragment of the *Sarcocystis* spp. 18S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR) using the primers SarcoFext and SarcoRext as previously described [4]. Amplification products (with an estimated concentration of at least 40 ng/µl) were purified using a commercial kit (Wizard SV clean upsystem, Promega) according to manufacturer instructions, and submitted for sequencing to the Genomic Unit, Biotechnology Institute CICVyA – CNIA –INTA, Argentina with both primers mentioned above. Sequences obtained were aligned and analyzed using the Geneious software (R9 version). Consensus sequences obtained were compared with others reported in GenBank by BLASTn analysis. Samples with inconclusive amplicon sequencing results were considered as potential mixed infections of different protozoan species [14]. Only assembled sequences with high quality chromatograms were submitted to GenBank (accessions numbers listed in Table 1). Sequences with a BLASTn identity ≥99% were recorded with a specific species name and the ones with ≤98% of sequence identity with other sequences deposited in GenBank as well as sequences that showed similar identity with more than one previously reported species were registered as *Sarcocystis* sp.

3. Results

From the total of Pampas foxes analyzed, 23 (17.6%; 10 females and 13 males) had *Sarcocystis* spp. oocysts/sporocysts in fecal samples and/or mucosal samples. *Sarcocystis* spp. sporocysts were detected in 13.0% (17/131) of fecal samples and in 39.4 % (13/33) of mucosal samples by the initial sugar flotation (Fig. 1A-B). The ellipsoidal sporocysts in feces measured 15 × 9 µm (18 – 10.5 x 10 – 8 µm) (n=17). Out all the animals with both samples analyzed (fecal and mucosal scraping), 6/33foxes were positive both samples, 7/33 were only positive in mucosal scraping and one was only positive in the fecal sample. Additionally, one fecal sample showed small spherical oocysts (10-12 µm diameter) compatible with *Neospora caninum/Hammondia heydorni* oocysts and was also processed by DNA extraction, PCR and sequencing.

A total of 21 *L. gymnocercus* samples (3 positive fecal samples, could not be processed) were processed by DNA extraction and PCR, 8 from the fecal samples, 11 from the mucosal scraping and 2 from both samples (Table 1). All these samples were positive in PCR and 23 sequencing reactions were performed (21 animals and 2 from both samples) (Table 1). The Table 1 shows the results of sequencing and BLASTn comparisons as well as the GenBank accession numbers of *Sarcocystis* spp., *Eimeria* spp., *Cystoisospora* spp. and *H. heydorni* 18S rRNA sequences obtained from all *L. gymnocercus* samples. Considering the mixed chromatograms and the identification of other protozoans, 7 foxes harbored mixed protozoan infections (Table 1). A total of sixteen 18S rRNA gene sequences were registered in the GenBank under the accession numbers: KY614531-KY614546 (Tables 1 for detailed accession numbers). Of those, two sequences correspond to a same Pampa fox (one from the fecal sample and other from mucosal material). The Figure 2 summarizes the number of *L. gymnocercus* samples with *Sarcocystis* spp. molecular identification.

Molecular identification of *Sarcocystis* spp. infection was successfully achieved in 14 foxes and was distributed as follows: 4.6% *S. cruzi* (6/131; single infection), 3.8% *Sarcocystis* spp. using birds as IH (*S. albifronsi* (EU502868) and *S. anasi* (EU553477) among others; 5/131; 4 single infections and 1 coinfection), 0.8% *S. tenella* (1/131; single infection) and 1.5% (2/131, single infections) with low homology (97%) with *S. miescheriana*. In the fecal sample with spherical oocysts, the sequencing results showed a 100% sequence identity with *H.heydorni* 18S rRNA gene sequences (Table 1).

4. Discussion

The majority of the investigations carried out in South America revealed a prevalence lower than 20% for *Sarcocystis* spp. sporocysts in domestic canids [e. g. 11, 15, 16], but a high prevalence (42-72%) was found in sheepdogs of Perú [17]. Moreover, there is still lack of investigations into the presence of oocysts/sporocysts in fecal or the intestinal tract of naturally infected wild canids of South America.

The overall prevalence of *Sarcocystis* spp. oocysts/sporocysts detected in *L. gymnocercus* was 17.6%. However, considering the higher sensitivity of the mucosal scraping versus fecal samples (39.4% versus 13%); the real prevalence is probably the detected in the mucosal scraping samples. In addition, out all animals with fecal and mucosal scraping samples, only 6 were positive in both and 7 only positive in the mucosal scraping sample. These results suggest that more than double *Sarcocystis*

spp. oocysts/sporocysts prevalence should be expected in mucosal scraping than in fecal samples. This matches with previous comparative studies performed with fecal and intestinal mucosal samples from infected DH, which revealed a higher proportion of oocysts/sporocysts in mucosa-derived material than in fecal samples [e. g. 1, 3]. Altogether, reinforces that oocysts are concentrated in the *lamina propria* of the small intestine and that sporocysts are released only sporadically during the course of the infection [1]. Taking into account the results obtained in mucosal scrapings, the prevalence found in *L. gymnocercus* was higher (39%) than in domestic dogs.

The primers used in the present study, targeting a fragment of the 18S rRNA gene from several *Sarcocystis* spp. but also from other related coccidian. Therefore, samples that had shown inconclusive results in sequencing were considered as putative mixed infections [3, 14, 18]. Regarding species identification, only sequences with a BLASTn identity≥99% were considered as species specific and the respective sequence entered into GenBank along with the respective species name. Sequences with lower identity with other reported sequences by BLAST as well as sequences that showed similar identity with more than one previously reported species were submitted to GenBank as '*Sarcocystis* sp.'. The DNA samples obtained from mucosal scrapings showed higher products concentration on PCR (data not shown) and further produce the better sequencing results.

Out all samples, 7 samples showed mixed chromatograms (n=5) or *Eimeria* spp./*Cystoisospora* spp. sequence identities (n=2) and the results were not reported on the GenBank (Table 1). Considering that in all these samples, *Sarcocystis* spp. oocysts/sporocysts have been observed, they were identified as mixed protozoan infections. Cloning the amplicons could be an efficient tool to identify properly these mixed infections, and probably more than one *Sarcocystis* spp. could be present at the same time in a single DH [3]. However due to economic factors we were not able to proceed with the cloning as in previous studies. Additionally, it is also plausible that the *Eimeria* spp. sequences obtained correspond to passage material and not to an specific infection in the foxes.

Out of all sequences of *L. gymnocercus* analyzed in this work, the most often identified *Sarcocystis* spp. were *S. cruzi* (4.6%) and *Sarcocystis* spp. using birds as IH (3.8%); followed by *Sarcocystis* sp. (1.5%) (*S.miescheriana* like =97% sequence identity) and *S. tenella*(0.8%).

Sarcocystis cruzi is the most frequent species infecting cattle (as IH) worldwide. Moreover, over than 90% of adult cattle have been found infected in some countries including Argentina [1, 19]. This species is transmissible via dogs, coyotes, foxes, wolves, and raccoons. Similar studies conducted from foxes and raccoon dogs in Europe evidenced lower prevalence or even no detection of *S. cruzi* in those carnivores [3, 5]. The relatively high frequency detected in Pampas foxes could be associated with a higher prevalence detected in cattle samples from Argentina versus European countries like Germany [7, 18]. The correlation of the concentration of the prey (cattle) in a given region could lead to increase the possibilities of foxes to feed on death or slaughtered animals. This report suggests that the *L. gymnocercus* is a DH for *S. cruzi*. Therefore, *L. gymnocercus* play an important role in the epidemiology this protozoan infection, and suggests that other cattle-canids transmitted parasitic infections could be cycling in the same area (i. e. Hidatidosis, Neosporosis, etc.).

The second *Sarcocystis* spp. identified in *L. gymnocercus* were species with birds as intermediate hosts. The detection could represent one or more of the 30 *Sarcocystis* species described in avian species, some of them using other canids as definitive hosts [20]. Four *Sarcocystis* species are described in birds of the order Anseriformes (i.e. *S.anasi*, *S.albifronsi, S.wobeseri*, *S. rileyi*) [21, 20, 22]. A trustworthy molecular identification is normally difficult for these species, since a highly conserved 18S rRNA gene sequence has been reported among *Sarcocystis* spp. producing cysts in different avian species and a high identity (≥98%) with *S. neurona*, *S. canis* and *S. mucosa* has been described by BLAST comparison [1, 22]. Therefore, it is advisable for a reliable specific identification, other gene targets like ITS1, cox1 or microsatellite markers should be applied in samples suspected to contain DNA of such *Sarcocystis* spp. [1, 2, 5] .According that the study was conducted with mucosal scrapings and oocysts were observed, the most suitable hypothesis is that Pampas foxes acted as DH, however, the potential "passage" of parasites from smaller carnivores-omnivores ingested by foxes could not be totally excluded. Several avian species represent an important dietary item of *L. gymnocercus* [9]; therefore it is possible that *Sarcocystis* spp. cycling among birds and foxes occurs frequently.

Sarcocystis tenella was identified in one sample, suggesting that sheep is infrequently prey by *L. gymnocercus*. This results contrast with the previous studies conducted from foxes in Europe. Sarcocysts in sheep muscles are microscopic and the infection could cause abortion and productive lost in sheep production [23]. In Brazil, *Sarcocystis*-associated mortality was reported in sheep [24], but the species was not identified. Until now, there is no report of *S. tenella* infecting sheep in Argentina. Therefore, the presence and confirmation of *S. tenella* in an intestinal mucosa sample confirm the presence of the species in Argentina and suggests *L. gymnocercus* as a potential DH.

Sarcocystis miescheriana uses wild boar and domestic pigs as IH [1]. In contrast with the study conducted with fox and raccoon dog samples in Germany, *S. miescheriana* was not detected in *L. gymnocercus* samples [3]. These results suggests that while European foxes prey mostly on pigs and wild boards, the Pampas fox did not preferred or have no contact with swine tissues. On the other hand, a *S. miescheriana*-like sequence was detected in 2 samples. The low sequence identity (97%) observed, could represents a new *Sarcocystis* species or/and a known species but from which is not sequence reported. The first assumption appears to be the most probable, since a highly similar sequence was obtained from sarcocysts of an unnamed species in muscles of an *armadillo* (*Chaetophractus villosus*) (Moré et al., unpublished). Species description and confirmation of life cycle requires further investigation.

Additionally, *Hammondia heydorni* was identified in one Pampas fox sample. *Hammondia heydorni* and *Neospora caninum* are closely related tissue-cyst forming members of the Sarcocystidae family. Both parasites use domestic and wild canids as DH and a wide variety of herbivore species as IH. Clinically, *N. caninum* holds more importance since is cause of abortions in cattle and neuromuscular disease in young dogs, while *H. heydorni* generally does not produce clinical signs in either hosts [25]. Morphologically, oocysts of these two parasites are indistinguishable and only the advent of molecular techniques and bioassays has allowed conclusive identification [26]. The prevalence of *H. heydorni* in dog populations appears to be low, with large-scale studies detecting

prevalence rates of around 0.2% [27, 28]. Comparatively little is known about the ecology of *H. heydorni* in wildlife and few studies conducted molecular identifications [29].The detection rate of *H. heydorni* DNA in *L. gymnocercus* in this study is similar to previous reports in others wild canids [29, 30] and is also similar to the detected in domestic dogs. Therefore, *L. gymnocercus* is suggested as a natural DH of *H. heydorni* in Argentina and future studies should be conducted in order to confirm the presence of the closely related protozoa *N. caninum*.

Our study presents a molecular approach to identify *Sarcocystis* spp. infections in the intestinal mucosa and fecal samples from *L. gymnocercus*. The results show that the mucosal scrapings are the eligible sample to identify prevalence and to proceed with species identification. *Lycalopex gymnocercus* is suggested as DH for *S. cruzi*, *S. tenella* and probably various *Sarcocystis* spp. using birds as intermediate hosts as well as for *H. heydorni*. Additionally, *Sarcocystis* spp. cycling between wild animals (Pampas foxes and armadillos) are partially identified and requires further investigation. As a final comment, we considered that *Sarcocystis* spp. identification is an important tool for the study of trophic interactions in wild hosts.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgements

We would like to thank the Municipal Zoonosis Center from General Pueyrredón for providing the space to develop our lab activities. This work was supported by grants PIP N029/11 and PIP 090 (*Consejo Nacional de Investigaciones Científicas y Técnicas,* Argentina), and EXA 668/14 (*Universidad Nacional de Mar del Plata*, Argentina).

References

[1] J.P. Dubey, R. Calero-Bernal, B.M. Rosenthal, C.A. Speer, R. Fayer, Sarcocystosis of Animals and Humans. CRC Press, Boca Raton, Florida (2015) pp 510.

[2] B. Gjerde, Phylogenetic relationships among *Sarcocystis* species in cervids, cattle and sheep inferred from the mitochondrial cytochrome c oxidase subunit I gene. Int. J. Parasitol. 43 (2013) 579–591.

[3] G. Moré, A. Maksimov, F.J. Conraths, G. Schares. Molecular identification of *Sarcocystis* spp. in foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) from Germany. Vet. Parasitol. 220 (2016) 9–14.

[4] G. Moré, S. Schares, A. Maksimov, F.J. Conraths, M.C. Venturini, G. Schares, Development of a multiplex real time PCR to differentiate *Sarcocystis* spp. affecting cattle. Vet. Parasitol.197 (2013) 85–94.

[5] P. Prakas, S. Liaugaudaite, L. Kutkiene, A. Sruoga, S. Svazas, Molecular identification of *Sarcocystis rileyi* sporocysts in red foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) in Lithuania. Parasitol. Res. 114 (2015) 1671–1676.

[6] M.B. Chhabra, K. Muraleedharan. Parasitic Zoonoses and Role of Wildlife: An Overview. Vet. Res.4 (2016) 01–11.

[7] G. Moré, D. Bacigalupe, W. Basso, M. Rambeaud, M.C. Venturini, L. Venturini, Serologic profiles for *Sarcocystis* sp. and *Neospora caninum* and productive performance in naturally infected beef calves. Parasitol. Res.106 (2010) 689–693. ISSN: 0932-0113

[8] A.A. Farias, M.J. Kittlein, Small-scale spatial variability in the diet of pampas foxes (*Pseudalopex gymnocercus*) and human-induced changes in prey base. Ecol. Res. 23 (2008) 543–550. doi: 10.1007/s11284-007-0407-7

[9] D. Canel, N.P. Scioscia, G.M. Denegri, M. Kittlein, Dieta del zorro gris pampeano (*Lycalopex gymnocercus*) en la provincia de Buenos Aires. Mastozool. Neotrop. 23 (2016) 359–370.

[10] S. Altizer, C.D. Harvell, E. Friedle, Rapid evolutionary dynamics and disease threat to biodiversity. Trends. Ecol. Evol.18 (2003) 589–596.

[11] S.V. Soriano, N.B. Pierangeli, I. Roccia, H.F.J. Bergagna, L.E. Lazzarini, A. Celescinco, M.S. Saiz, A. Kossman, P.A. Contreras, C. Arias, J.A. Basualdo, A wide diversity of zoonotic intestinal parasites infects urban and rural dogs in Neuquén, Patagonia, Argentina. Vet. Parasitol. (2010) 167 (1) 81-85.

[12] N.P. Scioscia, P.M. Beldomenico, G.M. Denegri, Contrastación de un Programa de Investigación Científica en Parasitología: los endoparásitos del zorro gris pampeano *Lycalopex gymnocercus*, Fil. Hist. Biol. (Brasil). 11 (2016) 107–120.

[13] E.A. Benbrook, M.W. Sloss, Parasitología Clínica Veterinaria. Compañía-Editorial Continental, México. (1965).

[14] G. Moré, N. Pantchev, D.C. Herrmann, M.G. Vrhovec, S. Ofner, F.J. Conraths, G. Schares, Molecular identification of *Sarcocystis* spp. helped to define the origin of green pythons (*Morelia viridis*) confiscated in Germany. Parasitology. 141 (2014) 646–651.

[15] T.C.G. Oliveira-Sequeira, A.F.T. Amarante, T.B. Ferrari, L.C. Nunes, Prevalence of intestinal parasites in dogs from Sao Paulo State, Brazil. Vet. Parasitol. 103 (2002) 19–27.

[16] J. López, K. Abarca, P. Paredes, E. Inzunza, Parásitos intestinales en caninos y felinos con cuadros digestivos en Santiago, Chile: Consideraciones en Salud Pública. Rev. Med. Chile. 134 (2006) 193–200.

[17] M. Choque, V. Chávez, P. Pacheco, V. Leyva, L. Panez, S. Ticona, Frecuencia de *Sarcocystis* sp. en perros pastores de asociaciones alpaqueras de Maranganí, Cusco. Rev. Investig. Vet. Perú. 18 (2007) 84–88.

[18] G. Moré, A. Pantchev, J. Skuballa, M.C. Langenmayer, P. Maksimov, F.J. Conraths, M.C. Venturini, G. Schares, *Sarcocystis sinensis* is the most prevalent thick-walled *Sarcocystis* species in beef on sale for consumers in Germany. Parasitol. Res. 113 (2014) 2223–2230.

[19] G. Moré, P. Abrahamovich, S. Jurado, D. Bacigalupe, J.C. Marin, M. Rambeaud, L. Venturini, M.C. Venturini, Prevalence of *Sarcocystis* spp. in Argentinean cattle. Vet. Parasitol. 177 (2011) 162–165.

[20] L. Kutkienė, P. Prakas, A. Sruoga, D. Butkauskas, Description of *Sarcocystis anasi* sp. nov. and *Sarcocystis albifronsi* sp. nov. in birds of the order Anseriformes. Parasitol. Res. 110 (2012) 1043–1046. doi: 10. 1007/s00436-011-2588-9

[21] L. Kutkienė, P. Prakas, A. Sruoga, D. Butkauskas, The mallard duck (*Anasplatyrhynchos*) as intermediate host for *Sarcocystis wobeseri* sp. nov. from the barnacle goose (*Brantaleucopsis*). Parasitol. Res. 107 (2010) 879–888. doi: 10.1007/s00436-010-1945-4

[22] L. Kutkienė, P. Prakas, A. Sruoga, D. Butkauskas, Identification of *Sarcocystis rileyi* from the mallard duck (*Anas platyrhynchos*) in Europe: cyst morphology and results of DNA analysis. Parasitol. Res. 108 (2011) 709–714. doi: 10.1007/s00436-010-2117-2

[23] R.G. Leek, R. Fayer, A.J. Johnson, Sheep experimentally infected with *Sarcocystis* from dogs. I. Disease in young lambs. J. Parasitol. 63 (1977) 642–650.

[24] C.A. Pescador, L.G. Corbellini, P.M. Bandarra, P.M.O. Pedroso, E.C.D. Oliveira, J.D.S. Leal, D. Driemeier, Aborto ovino associado com infecção por *Sarcocystis* sp. Pesq. Vet. Bras. Rio de Janeiro, RJ. 27 (2007) 393–397.

[25] J.P. Dubey, G. Schares, Neosporosis in animals-The last five years. Vet. Parasitol. 180 (2011) 90–108.

[26] A. Ash, A. Elliot, R.A. Thompson. The detection and characterization of *Neospora/Hammondia*-like oocysts from naturally infected dogs within the same urban region of Australia. Vet. Parasitol: Regional Studies and Reports, 1 (2015) 47–50.

[27] J.R. Slapeta, B. Koudela, J. Votypka, D. Modry, R. Horejs, J. Lukes, Coprodiagnosis of *Hammondia heydorni* in dogs by PCR based amplification of ITS-1 rRNA: Differentiation from morphologically indistinguishable oocysts of *Neospora caninum*. Vet. J. 163 (2002) 147–154.

[28] G. Schares, N. Pantchev, D. Barutzki, A.O. Heydorn, C. Bauer, F.J. Conraths, Oocysts of *Neospora caninum*, *Hammondia heydorni*, *Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. Int. J. Parasitol. 35 (2005) 1525–1537.

[29] D. Sinnott, K.M. Torres, B. Wolfe, R. Garabed, A.E. Marsh. Detection of *Hammondia heydorni* DNA in feces collected in and around an Ohio Wildlife Conservation Center. Vet. Parasitol: Regional Studies and Reports. 6 (2016) 31–34.

[30] S.A. Elmore, L.F. Lalonde, G. Samelius, R.T. Alisauskas, A.A. Gajadhar, E.J. Jenkins, Endoparasites in the feces of arctic foxes in a terrestrial ecosystem in Canada. Int. J. Parasitol. Parasites Wildl. 2 (2013) 90–96.

Figure legends

Figure 1A, B. Light micrographs of *Sarcocystis* spp. oocysts/sporocysts from naturally infected *Lycalopex gymnocercus*. Sporocysts from sample fecal (A) and oocysts/sporocysts from the small intestine mucosa (B) of Pampas fox. Scale bar = 20µm

Figure 2. Number of samples with *Sarcocystis* spp. identified by using molecular tools in mucosal material isolated from the small intestines and in fecal samples of *Lycalopex gymnocercus*. Results are shown for 18S rRNA gene sequences with an identity ≥ 99% and 97% with species-specific sequences deposited in GenBank.

Table

Table 1.

References: F: female. M: male. NR: not registered. (#) = accession numbers of sequences reported to GenBank. Mixed = no consensus sequence obtained or mixed chromatograms.

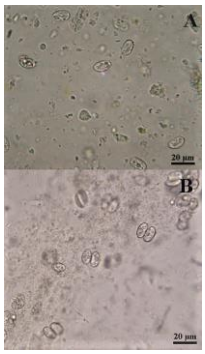


Fig. 1

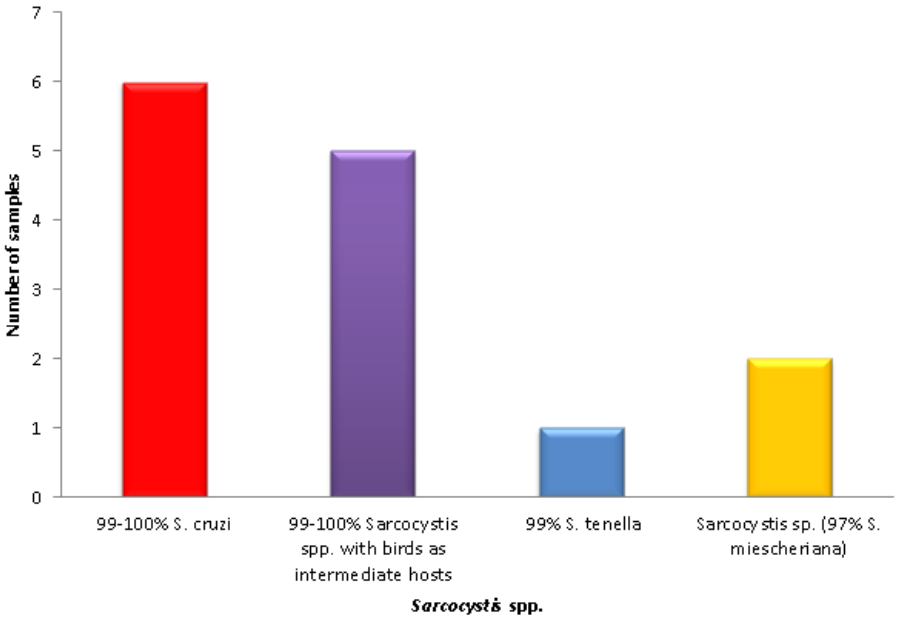


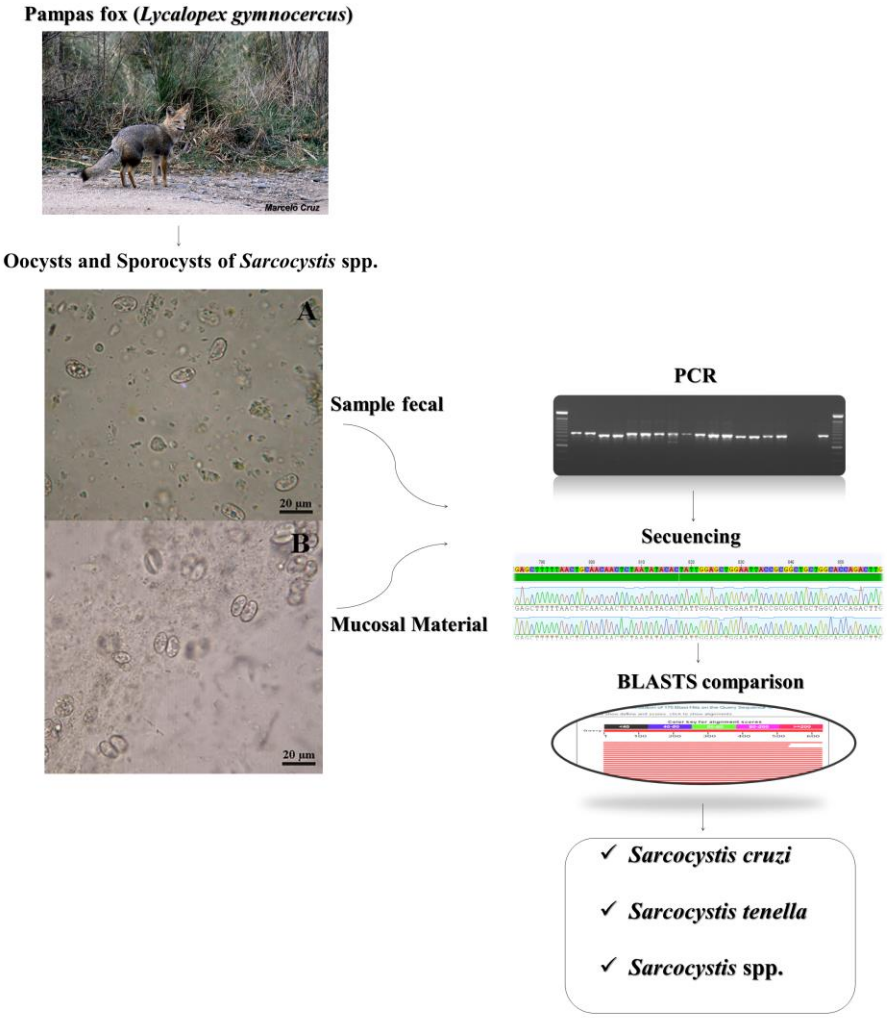
Fig. 2

Table 1. Consensus sequences of 18S rRNA gene fragments amplified from DNA extracted from small intestine mucosal scrapings (MS) and/or from fecal samples of *Lycalopex gymnocercus* containing microscopically visible Sarcocystidae oocysts/sporocysts.

Sample ID	Sex	Type of sample for	Molecular analysis		
		DNA extraction	Sequence base pairs	BLASTn identity (#)	*Sarcocystis* spp. (GenBank accession number)
102	**F**	**Fecal**	Mixed	NR	NR
139	**M**	**Fecal**	827	100% *S. cruzi* (KC209738, KT901167 and others)	*S. cruzi* (KY614531)
141	**M**	**Fecal**	Mixed	NR	NR
143	**M**	**Fecal**	810	100% *Sarcocystis* sp. (KT873792), *S. albifronsi* (EU502868) and *S. anasi* (EU553477)	*Sarcocystis* sp. (KY614532)
149	**F**	**Fecal**	833	100% *S. cruzi* (KC209738 y otras)	*S. cruzi* (KY614533)
154	**M**	**Fecal**	Mixed	NR	NR
162	**F**	**Fecal**	254	100% *Cystoisospora* spp. (AY618555, KU198329, KT184368 and others)	NR
191	**M**	**Fecal//MS**	754 // 813	99% *Eimeria nafuko* (JQ993665) and *E. falciformis* (KT184340)// *Sarcocystis* sp. (KT873792), *S. albifronsi* (EU502868) and *S. anasi* (EU553477)	NR//*Sarcocystis* sp. (KY614534)
202	**M**	**MS**	Mixed	NR	NR
205	**M**	**Fecal// MS**	768 // 815	100% *Sarcocystis* sp. (KT873792), *S. albifronsi* (EU502868) and *S. anasi* (EU553477) // 99% *Sarcocystis* sp. (KT873792), *S. albifronsi* (EU502868) and *S. anasi* (EU553477)	*Sarcocystis* sp. (KY614535)//*Sarcocystis* sp. (KY614536)
208	**F**	**MS**	797	99% *S. tenella* (KC209734, KP263759 and others)	*S. tenella* (KY614537)

212	**F**	**MS**	786	100% *S. cruzi* (KC209738, KT901167 and others)	*S. cruzi* (KY614538)
217	**M**	**MS**	808	97% *S. miescheriana* (KT873760, JN256123 and others)	*Sarcocystis* sp. (KY614539)
224	**F**	**MS**	829	97% *S. mieschariana* (KT873760, JN256123 and others)	*Sarcocystis* sp. (KY614540)
290	**F**	**MS**	811	100% *S. cruzi* (KC209738, KT901167 and others)	*S. cruzi* (KY614541)
291	**F**	**MS**	745	99% *Sarcocystis* sp. (KT873792), *S. albifronsi* (EU502868) and *S. anasi* (EU553477)	*Sarcocystis* sp. (KY614542)
292	**M**	**MS**	Mixed	NR	NR
293	**F**	**MS**	798	100% *S. cruzi* (KC209738, KT901167 and others)	*S. cruzi* (KY614543)
294	**F**	**MS**	772	99% *Sarcocystis* sp. (KT873792), *S. albifronsi* (EU502868) *and S. anasi* (EU553477)	*Sarcocystis* sp. (KY614544*)*
297	**F**	**Fecal**	746	100% *Hammondia heydorni* (KT184370, JX220987 and others)	*H. heydorni like* (KY614545)
298	**M**	**MS**	244	100% *S. cruzi* (JX679467, KT964019 and others)	*S. cruzi* (KY614546)

References: F: female, M: male. NR: not registered. (#) = accession numbers of sequences reported to GenBank. Mixed = no consensus sequence obtained or mixed chromatograms.



Graphical abstract

Highlights

- Occurrence of *Sarcocystis* spp. in mucosal scrapings of the small intestine (n=33) and fecal samples (n=131) of *Lycalopex gymnocercus*.
- *Sarcocystis* spp. (oocysts and sporocysts) were detected in fecal samples and/or mucosal samples of 17.6% of *L. gymnocercus*.
- *Sarcocystis* spp. sporocysts were detected in 13.0% of fecal samples and in 39.4 % of mucosal samples by the initial sugar flotation.
- PCR and sequencing of 18S rRNA gene was suitable to identify several *Sarcocystis* spp.
- *Lycalopex gymnocercus* is suggested as definitive host for *S. cruzi*, *S. tenella*, *Hammondia heydorni* and *Sarcocystis* spp. using birds as intermediate hosts.